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Protection with recombinant *Clostridium botulinum* C1 and D binding domain subunit (Hc) vaccines against C and D neurotoxins

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Abstract

Recombinant botulinum Hc (rBoNT Hc) vaccines for serotypes C1 and D were produced in the yeast *Pichia pastoris* and used to determine protection against four distinct BoNT C and D toxin subtypes. Mice were vaccinated with rBoNT/C1 Hc, rBoNT/D Hc, or with a combination of both vaccines and challenged with BoNT C1, D, C/D, or D/C toxin. Mice receiving monovalent vaccinations were partially or completely protected against homologous toxin and not protected against heterologous toxin. Bivalent vaccine candidates completely survived challenges from all toxins except D/C toxin. These results indicate the recombinant C1 and D Hc vaccines are not only effective in a monovalent formula but offer complete protection against both parental and C/D mosaic toxin and partial protection against D/C mosaic toxin when delivered as a bivalent vaccine.

Keywords: Botulinum neurotoxin subtypes; Recombinant vaccine; Receptor-binding domain

1. Introduction

Botulism is the collective term for the neurophysiological effects caused by clostridial neurotoxins. There are seven serologically unique types of *Clostridium botulinum* neurotoxins, denoted BoNT A–G, that consist of a 150-kDa holotoxin with an N-terminal 50-kDa light chain (LC) and a C-terminal 100-kDa heavy chain (HC), linked by a single disulfide bond [1]. The BoNT HC is further delineated into the N-fragment, or translocation domain (Hn), and the C-fragment, or receptor-binding domain (Hc), forming three functional domains that mediate intoxication of the neuron in a defined pathway. The toxin is initially introduced into cholinergic nerve cells by receptor-mediated endocytosis involving the interaction of the Hc domain with specific receptors [2]. The acidic pH of the endosome is believed to initiate a conformational change in the dichain toxin that results in the formation of a protein channel by the Hn through which the LC is translocated out of the endosomal lumen and into the cytosol [3]. The LC is a zinc-dependent, endoprotease that cleaves SNARE proteins which are critical for vesicular trafficking and release of neurotransmitter at cholinergic synapses [2].

Because of the relatively high amino acid sequence heterogeneity of 32–60% of the neurotoxins, little, if any, cross-protection is seen among the serotypes, necessitating the development of vaccines against each individual serotype. BoNT C1 and D strains are unique among the neurotoxins in that their toxin genes are carried on bacteriophages [4]. This may contribute to the relative mobility of their toxic components and the diversity seen in C and D neurotoxin sequences.

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14. ABSTRACT
Recombinant botulinum Hc (rBoNT Hc) vaccines for serotypes C1 and D were produced in the yeast Pichia pastoris and used to determine protection against four distinct BoNT C and D toxin subtypes. Mice were vaccinated with rBoNT/C1 Hc, rBoNT/D Hc, or with a combination of both vaccines and challenged with BoNT C1, D, C/D, or D/C toxin. Mice receiving monovalent vaccinations were partially or completely protected against homologous toxin and not protected against heterologous toxin. Bivalent vaccine candidates completely survived challenges from all toxins except D/C toxin. These results indicate the recombinant C1 and D Hc vaccines are not only effective in a monovalent formula but offer protection against both parental and mosaic toxins when delivered as a bivalent vaccine.

15. SUBJECT TERMS
Clostridium botulinum, neurotoxin, subtypes, recombinant vaccine, receptor-binding domain

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BoNT C1 toxin was shown to produce a blockade of neuromuscular transmission in human pyramidalis neuromuscular junction preparations under identical experimental conditions as BoNT A toxin [5], and to inhibit exocytosis in cerebellar granule neurons over multiple weeks (BoNT C1 – 13 pM inhibits for >25 days; BoNT A – 10 pM inhibits for >31 days) [6]. BoNT C/D is currently being used to treat dystonia in humans [7]. Both BoNT C1 and BoNT D/C were lethal by aerosol to nonhuman primates [J. Anderson, personal communication]. This information taken together supports the belief that BoNT C and D, and their mosaic counterparts, may be potentially lethal in humans. We have been developing recombinant vaccines against BoNT believed to be potential bioterrorist or biowarfare threats.

On rare occasions BoNT C toxin or *C. botulinum* type C organisms have been associated with cases of foodborne botulism. Two cases of foodborne botulism linked to BoNT C and one case of infant botulism attributed to *C. botulinum* serotype C1 have been reported [8,9], and BoNT D organisms were found in tainted ham that caused mild botulism in several persons [9]. However, BoNT C1 and D are predominantly known for causing avian or mammalian botulism [10–12]. Limited stocks of anti-BoNT C1 and D veterinary vaccines are used to vaccinate cattle and other domestic animals, and they have been shown to be protective. For example, in a recent outbreak in foxes and minks in Finland, vaccinated animals showed a 1.5% overall death rate, where unvaccinated susceptible foxes had death rates of approximately 25% [12].

BoNT C1 from strains Stockholm and 468 share 99.9% nucleic acid identity [13,14]. Similarly, BoNT D from strains 1873 and CB-16 also share 99.9% DNA sequence identity [15,16]. However, as neurotoxins from more individual strains of BoNT C and D were characterized, some displayed an inconsistent reactivity to BoNT C and D antitoxins [17–20]. Initial serological data suggested that the bacteria might be encoding both BoNT C and D but DNA sequencing revealed the existence of mosaics that contain elements of both C and D holotoxins [21,22]. BoNT from strain C6813 (a C/D mosaic) has the sequence of BoNT C1 for the amino-terminal 2/3 of the protein (95% identity), but is 95% identical to BoNT D in the carboxy-terminal third [21]. Similarly, when the bacteriophage from the South African strain of BoNT D (Dsa) was sequenced, the LC was found to have a very high amino acid identity with BoNT/D LC (96%) and Hn (92%), but shared only 78% and 40% identity with the BoNT/C1 and /D Hc, respectively [22]. The sequence divergence observed in the receptor-binding domain of the BoNT Dsa (a D/C mosaic) appears to be unique among the BoNT toxins.

This dual antigenic nature of the C and D mosaic toxins presents obvious challenges to developing prophylactic treatments, including recombinant Hc subunit vaccines. Furthermore, D/C mosaic toxins present an even more unique problem due to amino acid sequence variation in the Hc that diverges significantly from either of the parental holotoxins.

In this study, mice were vaccinated with rBoNT/C1 Hc and rBoNT/D Hc, in monovalent and bivalent formulations, to compare protection against homologous and mosaic toxin strains with each vaccine, and with both vaccines in combination.

2. Materials and Methods

2.1. Construction of rBoNT Hc genes and transformation in Pichia pastoris

2.1.1. rBoNT/C1 Hc

A synthetic rBoNT/C1 Hc gene encoding amino acid residues Thr-843 to Glu-1291 (NCBI accession # BAA14235) was constructed using a *P. pastoris* alcohol oxidase 1 (*AOX1*) codon bias. N-terminal residues were chosen for stability consistent with the N-end rule governing protein expression in yeast. [23] (Fig. 1A). The rBoNT/C1 Hc open reading frame (ORF) was ligated into the *Eco*RI site of yeast expression vector pHILD4 (Philips Petroleum, Bartlesville, Oklahoma) and the plasmid construct was linearized with *Sac*I and introduced into the methylotropic yeast, *P. pastoris* GS115, by spheroplast transformation according to the manufacturer's specifications (Invitrogen, Carlsbad, CA). Recombinant yeast strains containing multiple copies of the insert DNA were selected by screening on yeast extract peptone dextrose (YPD) plates containing increasing amounts of G418 (geneticin sulfate). Despite the care taken in gene construction, the resulting protein from this construct was found to be truncated by 22 amino acids. Repeated attempts to produce full-length protein by adjustments in fermentation and purification parameters failed, so a new synthetic rBoNT/C1 Hc gene beginning at amino acid residue 22 of the original construct was made. The new construct, encoding amino acid residues Tyr-865 to Glu-1291 of rBoNT/C1, was ligated into the *Eco*RI site of yeast expression vector pPICZA (Invitrogen, Carlsbad, CA). The plasmid construct was linearized with *Sac*I and transformed into *P. pastoris* X-33. Clones with multi-copy inserts were selected on increasing amounts of Zeocin and confirmed by southern blot and a five-copy clone was selected based on performance under fermentation conditions.

2.1.2. rBoNT/D Hc

Similarly, a synthetic rBoNT/D Hc gene encoding amino acid residues Ala-830 to Glu-1276 (NCBI accession #BAA75084) was constructed using *AOX1* codon bias favoring yeast-stable N-terminal amino acids (Fig. 1B). The rBoNT/D Hc ORF was ligated into the *Eco*RI site of pPICZB (Invitrogen). The rBoNT/D Hc gene construct was linearized with *Sac*I and introduced into yeast *P. pastoris* strain X-33 by electroporation. Recombinant yeast strains containing multicopy inserts were selected on YPD plates with 800, 1000, and 1500 mg/l of Zeocin.

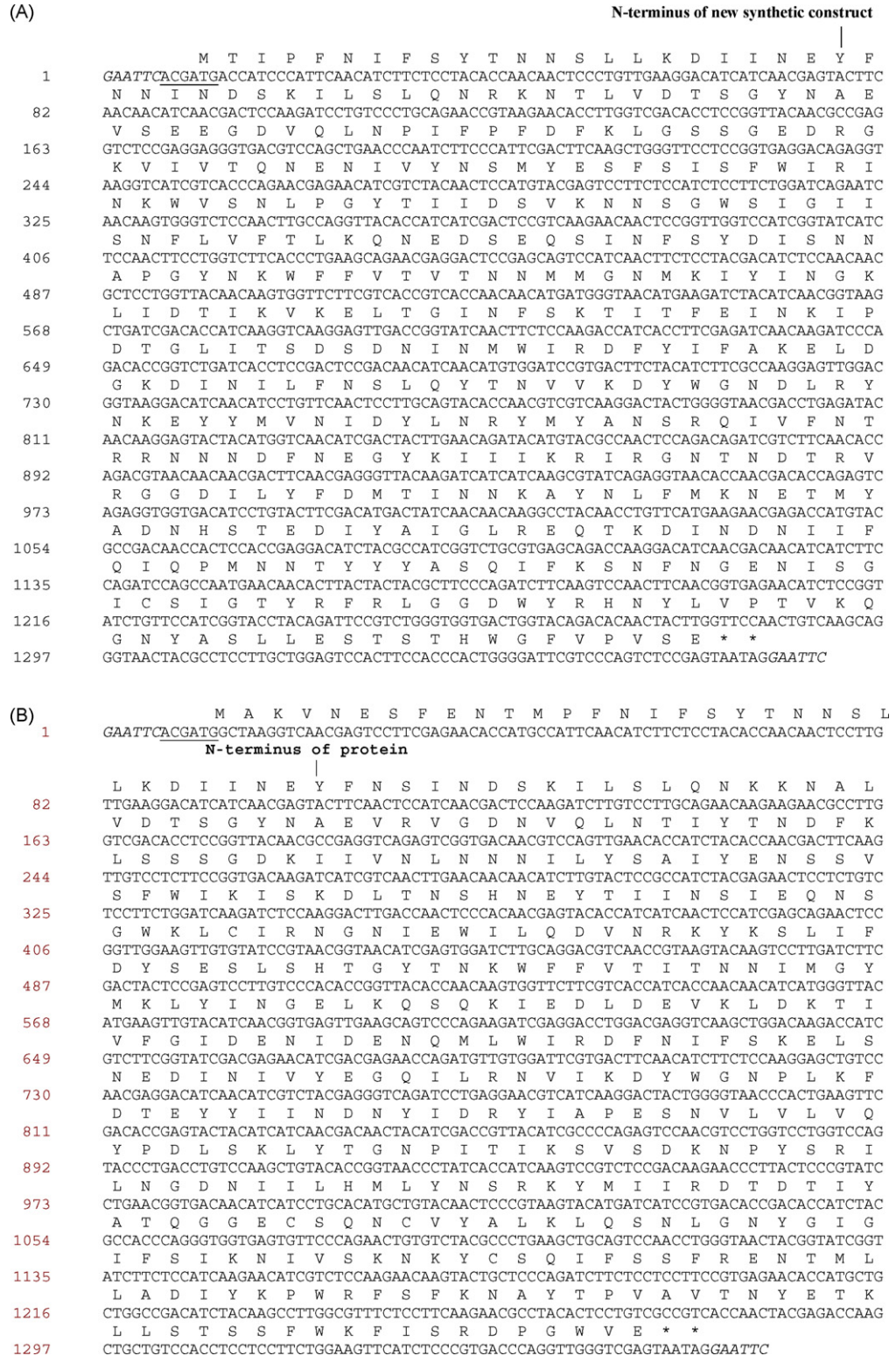

Fig. 1. Synthetic open reading frames (ORF) of the rBoNT/C1 Hc (A) and BoNT/D Hc (B) utilizing a *P. pastoris AOX1* codon bias. The *Eco*RI restriction sites are italicized, the core Kozak sequence is underlined.

2.2. Protein expression and purification

2.2.1. rBoNT/C1 Hc

Fermentations were performed as described elsewhere [24]. Purifications were done using a three-step process. The first step involved loading extract from the cell pellet, in 50 mM sodium phosphate, 2 M NaCl, pH 6.5, onto a MEP HyperCel column (Pall Corp., East Hills, NY) followed by multiple washes in succinate buffers of decreasing pH and elution using a gradient of various percentages of 50 mM succinate, pH 4.5 and 25 mM succinate, pH 3.0. The second step included a CM 650 M (weak anion exchange) column (Applied Biosystems, Foster City, CA), with initial loading and washing in 25 mM succinate, 650 mM NaCl, pH 4.0. Material was eluted from the column using a salt gradient of 650 mM–1 M NaCl in succinate, pH 4.0. The final step included a second MEP HyperCel column, with elution using a decreasing salt gradient from 500 to 300 mM NaCl, plus a decrease in pH from 4.0 to slightly above 3.0. This purified material was diafiltered into 15 mM succinate, pH 4.0, and analyzed by SDS-PAGE, Western blot, and N-terminal sequencing.

2.2.2. rBoNT/D Hc

Selected recombinant strains of *P. pastoris* were cultured in minimal glycerol medium (MGY) at 30 °C until an optical density of 2–3 at 600 nm (OD_{600}) was attained and then switched to minimal medium containing 0.5% methanol (MM) to induce expression.

A clear extract produced from frozen *P. pastoris* pellet harboring the target protein was loaded onto a column containing Phenyl Sepharose 650 M resin (TOSOH BioSciences, Montgomeryville, PA) equilibrated with 20 mM sodium acetate (NaOAc), pH 6.2, 2 M NaCl, 2 mM EDTA, 1 M PMSF. The rBoNT/D Hc was eluted with a decreasing salt gradient using 20 mM NaOAc pH 6.2, 2 mM EDTA, 1 mM PMSF as Buffer B. Fractions containing rBoNT/D Hc were pooled and dialyzed against 3 changes of 20 mM NaOAc, pH 5.2, 2 mM EDTA, 1 mM PMSF for 3 h. Dialyzed fractions were loaded onto a cation exchange column containing POROS HS20 resin (Applied Biosystems, Foster City, CA) which had been pre-equilibrated with 20 mM NaOAc, pH 5.2, 2 mM EDTA, 1 mM PMSF. Protein was eluted with an increasing salt gradient using 20 mM NaOAc, pH 5.2, 2 mM EDTA, 0.5 M NaCl as the second buffer. Fractions containing rBoNT/D Hc were pooled and analyzed by SDS-PAGE, Western blot, and N-terminal sequencing.

2.3. Challenge studies

2.3.1. Challenge toxins

Neurotoxins used in ELISA and challenge studies were obtained from the following strains: BoNT C1 NCTC 8264 (Centers for Applied Microbiology Research (CAMR) Porton Down, UK); BoNT C/D 003–9 and BoNT D CB-16 (Wako Chemicals, Richmond, VA); and BoNT D/C VPI 5995 (Metabiologics, Madison, WI). BoNT C1 was available only as pure neurotoxin; BoNT C/D and D only as toxin complex; and BoNT D/C was available both in pure and complexed form.

Specific activity of the toxins was determined in mice by duplicate intraperitoneal (i.p.) LD_{50} endpoint titrations, using two-fold dilutions ranging from 20 to 0.156 LD_{50}, based on preliminary lethality values received from the manufacturers. Published neurotoxin gene sequences were available for three of the four toxins used in this study. Sequences for toxin subtypes BoNT C1 Stockholm, BoNT C/D 003-9, and BoNT D CB-16 are available from PubMed (# D90210, AB200360, and S49407, respectively). The sequence for BoNT D/C VPI 5995 was provided by Karen Hill, Los Alamos National Laboratories. This gene sequence has been deposited into the NCBI database as accession number EF378947. Alignments and identity comparisons of BoNT genes and proteins were generated using Vector NTI Suite software (Invitrogen, Carlsbad, CA) (Fig. 2).

2.3.2. Vaccinations

Female Crl:CD-1 mice (Charles River, Raleigh, NC) were received at approximately 5 weeks of age. Antigen stocks of rBoNT/C1 Hc and rBoNT/D Hc were diluted to 50 μg/ml in 25 mM sodium succinate, 15 mM sodium phosphate, pH 5.0, with 5% mannitol with 0.2% Alhydrogel as adjuvant. Groups of 10 mice were vaccinated intramuscularly (i.m.) at 0, 4, and 8 weeks with 5 μg of either rBoNT/C1 Hc, rBoNT/D Hc, or a combination of 5 μg each of both antigens in a total volume of 0.1 ml. Two weeks after the final vaccination, mice were challenged i.p. with 100,000 mouse LD_{50} in total injected volumes of 0.1 ml of the appropriate toxin. All challenge toxins were in complexed form except BoNT C1, which was pure neurotoxin. Statistical analyses on survival rate were done using Fisher Exact tests. Animal studies were conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhere to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.3.3. Potency assays

Potency assays were done as previously described [1]. Briefly, groups of 10 mice were vaccinated once with three-fold decreasing amounts of antigen from 8.1 μg to 11 ng using 0.2% Alhydrogel as adjuvant. Vaccinations were i.m., 0.1 ml total dose. Three weeks after vaccination, mice were challenged i.p. with 1000 LD_{50} of appropriate toxin in total volumes of 0.1 ml. Toxins were all in complexed form, except BoNT C1, which was pure neurotoxin. Survival was monitored over 5 days, and results were tallied and subjected to probit analysis (SPSS, Chicago, Illinois).

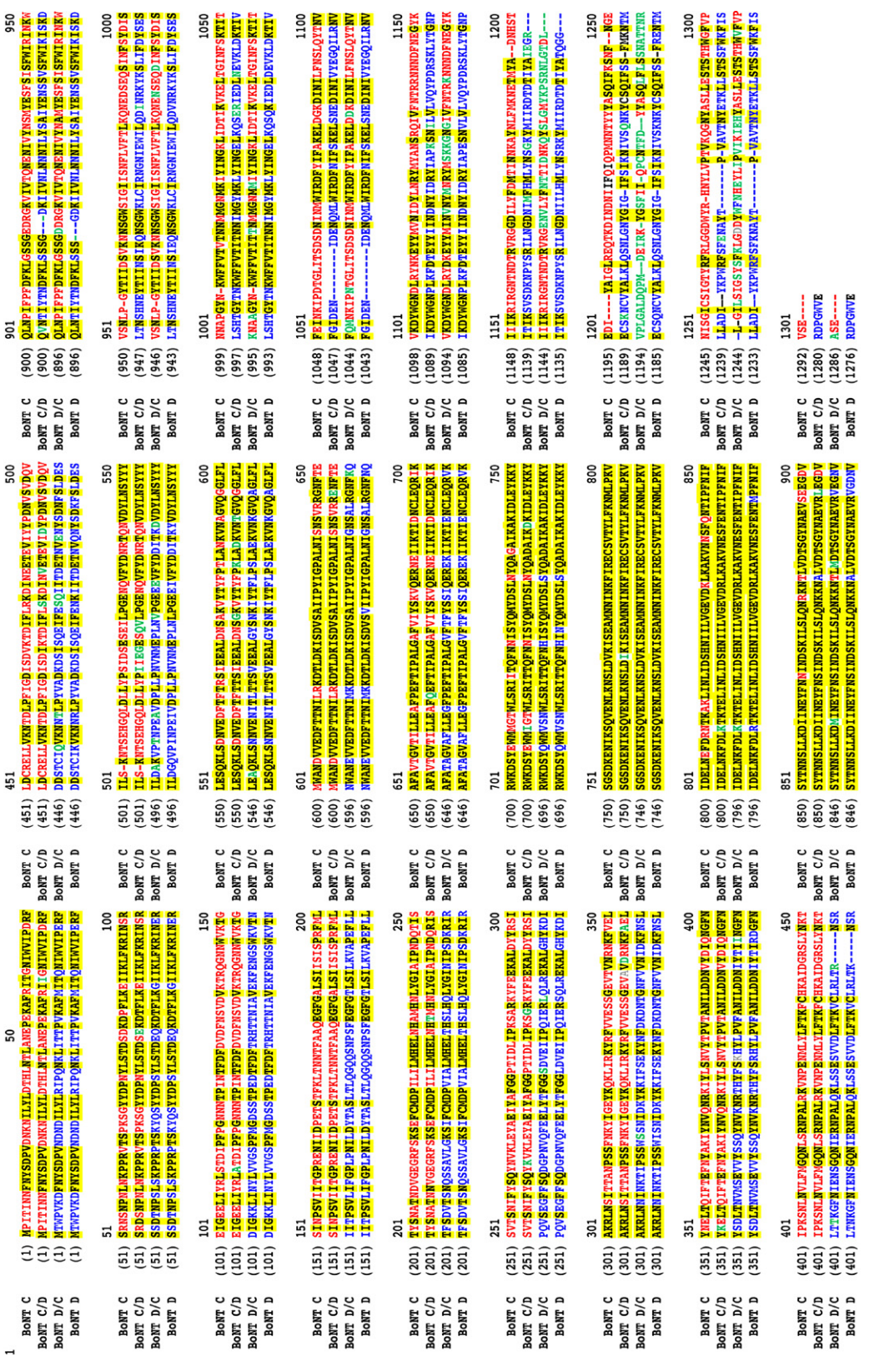

Fig. 2. Amino acid alignments of BoNTC, BoNT C/D, BoNT D/C and BoNT D showing mosaic nature of the C/D and D/C toxins. BoNT C sequences are red, BoNT D sequences are blue, conserved sequences are black with yellow highlighting, and sequences unique to BoNT D/C are green.

2.3.4. Mouse serum ELISA titers

Mice were bled 2 days prior to challenge to obtain serum for individual serum ELISA titers. Sera were tested in duplicate for reactivity against the same toxin type used for the mouse challenge. Ninety-six well microtiter plates were coated with toxins diluted to 2 μg/ml and incubated overnight at 4 °C. The toxins used to coat the plates were the complexed form of BoNT D and C/D or pure neurotoxin from types C1 and D/C. Skim milk diluent (5% skim milk in PBS, pH 7.4) was used to block nonspecific binding and as an antibody diluent. Plates were washed with PBS (pH 7.4) with 0.1% Tween-20 between steps. Test sera were initially diluted 1:100, followed by four-fold serial dilutions for a total of eight dilutions (1:100–1: 1,638,400), and incubated for 90 min at 37 °C. Goat anti-mouse antibody conjugated to horseradish peroxidase (KPL, Gaithersburg, MD), diluted 1:1000, was added as the secondary antibody, and incubated for 60 min at 37 °C. Plates were developed for 30 min at room temperature with 2, 2′-azino-di (3-ethylbenzthiazoline-6-sulfonate) (ABTS) (KPL, Gaithersburg, MD) and absorbance was read at 405 nm. The titer was defined as the reciprocal of the highest dilution with an absorbance ≥0.2 above background. Geometric means of the ELISA titers were generated to show overall comparisons in antibody development to each toxin subtype. Statistical analyses on geometric means were done using ANOVA with Tukey's post-hoc tests for comparisons between non-controls and Dunnett's post-hoc tests for comparisons to controls. Additional ELISAs comparing results using pure versus complexed BoNT D/C toxin were done (data not shown). Results were similar, regardless of toxin state.

3. Results

3.1. r BoNT/C1 Hc purification

While our original rBoNT/C1 Hc construct was designed specifically to produce stable full-length protein, the final product was found to contain truncations. The predominant protein species purified was 426 amino acid residues, representing a 22 amino acid deletion. The N-terminus of the protein consistently began with YFNNINDSKI. Modifications in fermentation and purification failed to produce full-length protein, and, as a homogeneous vaccine product is desirable and studies indicated that the additional 22 amino acid residues removed were not necessary for immunogenicity of the product, a new gene construct was made beginning at amino acid residue 865. The three-step purification scheme included initial capture using MEP HyperCel, followed by a weak anion exchange step that removed the majority of higher molecular weight *Pichia* proteins, and final removal of contaminants and any rBoNT/C1 Hc degradation products with a repeat of MEP HyperCel. Diafiltration was used to raise the pH and reduce the conductivity of the product. The final product had a concentration of 320 μg/ml and was >98% pure by visual inspection of an SDS-PAGE (Fig. 3) and Western blot (Fig. 4). Sequencing indicated the N-terminus of the protein was intact.

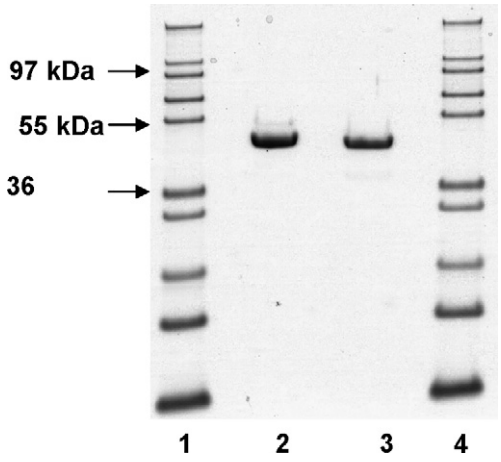

Fig. 3. SDS-PAGE of purified BoNT/C1 Hc and BoNT/D Hc. Lanes 1 and 4: molecular weight markers, lane 2–5 μg of rBoNT/C1 Hc, lane 3–5 μg of rBoNT/D Hc.

3.2. rBoNT/D Hc purification

The rBoNT/D Hc protein product was >95% pure, with a final concentration of 306 μg/ml. SDS-PAGE and Western blot analyses showed single bands of expected molecular weight. (Figs. 3 and 4). N-terminal sequencing of the purified D Hc vaccine revealed 95% of the purified polypeptide began with the sequence Y F N S I N D S K I, which represented a rBoNT/D Hc species lacking the first 31 amino acid residues from the expected N-terminus. Thus, the final rBoNT/D Hc protein consisted of 415 amino acid residues, from Tyr 861-Glu 1276. Care was taken to engineer the genes for these

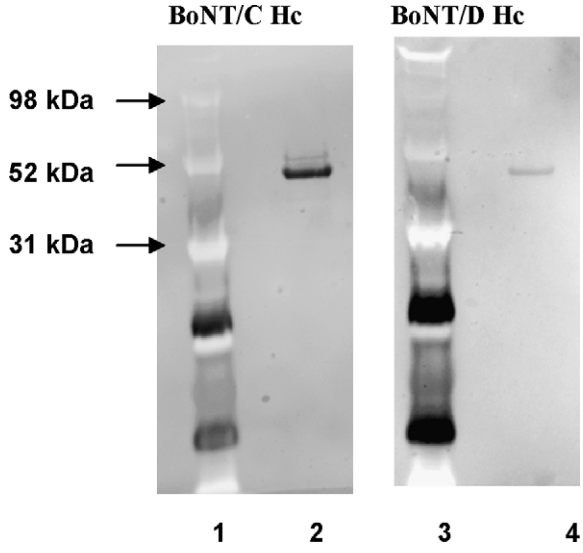

Fig. 4. Western blot of purified BoNT/C1 Hc and BoNT/D Hc. Lanes 1 and 3: molecular weight markers, lane 2: BoNT/C1 Hc, lane 4: purified BoNT/D Hc.

recombinant proteins to express full-length proteins, but, as with the rBoNT/C1 Hc protein, the rBoNT/D Hc showed a distinct but stable truncation.

3.3. Vaccination and challenge with BoNT C and D toxin subtypes

Mice receiving three vaccinations of 5 μg per mouse of either rBoNT/C1 Hc, rBoNT/D Hc, or a bivalent vaccine containing both antigens were challenged with 100,000 mouse LD_{50} of appropriate toxin. Survival results are presented in Table 1. Mice were challenged with BoNT C1 (NCTC 8264), BoNT C/D (003-9), BoNT D (CB-16), and BoNT D/C (VPI 5995) toxins. Neurotoxin from the VPI 5995 strain was thought to be type D, but previous studies showed that the toxin was not effectively neutralized by anti-D antitoxin and neurotoxin sequencing confirmed this toxin to be type D/C. Survival against challenges with BoNT C1 toxin after vaccination with rBoNT/C1 Hc or rBoNT/C1+D Hc was complete and partial survival was seen after challenge with BoNT D/C toxin. Complete survival was seen against challenges of either BoNT D or C/D toxin after vaccination with rBoNT/D Hc or rBoNT/C1+D Hc. Survival against D/C toxin was partial after vaccination with rBoNT/C1 Hc or rBoNT/C1+D Hc (3–4/10 mice).

Potency assays using BoNT/C1 and /D Hc against homologous toxin show good protection, with ED_{50} values of 109 ng (95% confidence limits = 54–208 ng) and 232 ng (95% confidence limits = 105–501 ng), respectively, after only one vaccination (Fig. 5). No mice survived challenge after vaccination with BoNT/D Hc and challenge with BoNT D/C toxin. As the efficacy results after three vaccinations with BoNT/C1 Hc at 5 μg/mouse and challenge with BoNT C/D toxin showed no survival, it was deemed unnecessary to run a one-dose potency assay using that vaccine-toxin combination.

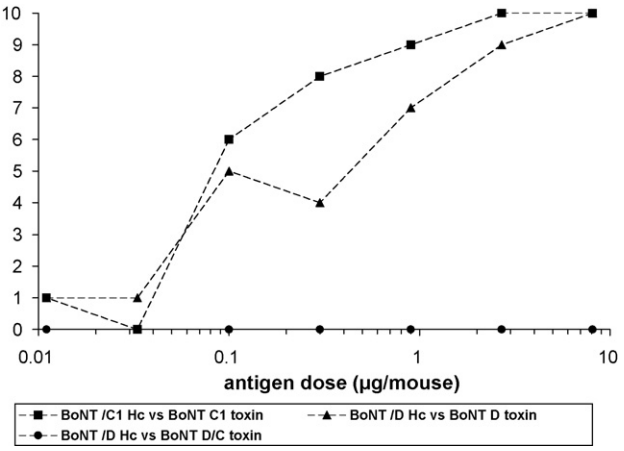

Fig. 5. Results from one-dose potency assays showing protection against homologous toxins. ED_{50} values for BoNT /C1 Hc vs. BoNT C toxin and rBoNT/D Hc vs. BoNT D toxin are 109 ng and 232 ng, respectively. There were no survivors when BoNT/D Hc-vaccinated mice were challenged with BoNT D/C toxin.

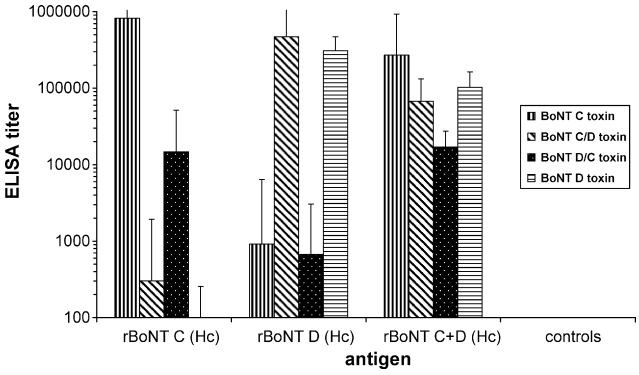

Fig. 6. Serum responses in mice following vaccination with rBoNT/C1 Hc, rBoNT/D Hc, or a combination of C1 and D Hc vaccines. Mouse sera were tested individually by ELISA where the antigens were the same subtype as the challenge toxins for those animals. Geometric means of the titers were generated for comparative purposes. Error bars represent the mean + 2 S.D.

3.4. Mouse serum ELISA results

Serum antibody titers were determined using individual serum ELISAs for each mouse against their challenge toxin. Geometric means of the titers are shown in Table 1 and Fig. 6. Mice vaccinated with rBoNT Hc antigen homologous to the challenge toxin Hc sequence developed the highest average titers, ranging from 67,559 to 819,200, with the exception of BoNT/D/C. In contrast, serum from mice vaccinated with rBoNT Hc heterologous to the Hc of the challenge toxin developed substantially lower titers ranging from 100 to 919. ELISA titers where BoNT D/C was used as coating toxin were 673 for rBoNT/D Hc-vaccinated mice, and only 14,703 and 16,890 for rBoNT/C1 or rBoNT/C1+D Hc-vaccinated mice, respectively. Overall differences in geometric mean ELISA titers were analyzed using ANOVA. As expected with

Table 1
Survival data and geometric means of mouse serum ELISA titers

	C NCTC 8264		C/D 003-9		D/C VPI 5995		D CB16	
	Challenge	ELISA	Challenge	ELISA	Challenge	ELISA	Challenge	ELISA
C (Hc)	10/10	819,200	0/10	303	4/10	14,703	0/10	100
D (Hc)	0/10	919	10/10	470,507	0/10	673	10/10	310,419
C (Hc) + D (Hc)	10/10	270,235	10/10	67,559	3/10	16,890	10/10	102,400
toxin controls	0/10	<100	0/10	<100	0/10	<100	0/10	<100

Mice were immunized 3 times at 0, 4, and 8 weeks with 5 μg per mouse of rBoNT/C1 Hc, rBoNT D Hc, or both antigens. Two weeks following final immunization, mice were challenged with 100,000 LD_{50} of listed toxin. Challenge numbers represent number of survivors/total number of mice in group.

the monovalent formulations, highly significant differences ($p < 0.0001$) were seen when comparing high versus low titers. However, differences when comparing high with high or low with low were not significant ($p = 0.5056$–0.9828), with the exception of mice vaccinated with rBoNT Hc where sera was tested against D/C toxin. Sequence differences between BoNT C and D/C in the Hc region were sufficient to lower antibody binding to significantly different levels ($p < 0.0001$). The BoNT D/C Hc region contains approximately 23% unique sequence that differs from any other serotype, including C1 and D. Thus, we would expect significant differences in ELISA titer after bivalent vaccination when comparing BoNT C, C/D, or D versus BoNT D/C toxin. Titers against BoNT C, C/D, or D ranged from 67,559 to 270,235 but averaged only 16,890 against BoNT D/C. These were significantly different levels ($p < 0.0001$–0.0354). In addition, differences between titers using C toxin versus C/D toxin were significant ($p = 0.0354$), but not when comparing C versus D or D versus C/D. These analyses indicate that rBoNT/C1 Hc is not a significantly better immunogen than rBoNT/D Hc, and that both antigens in combination are significantly less immunogenic against C/D mosaic toxins.

4. Discussion

There are currently no licensed vaccines for the prevention of botulism. Limited quantities of a pentavalent toxoid vaccine granted Investigational New Drug status in 1979 are available for individuals at risk of exposure. However, due to the difficulties and risks associated with producing toxoid vaccines [25] subsequent efforts have largely focused on recombinant vaccines for prophylaxis [26–29]. We have previously demonstrated that recombinant rBoNT Hc vaccines are highly efficacious, protecting against challenges of over 100,000 LD_{50} of toxin, often after a single vaccination [30].

However, botulinum neurotoxins are known to be diverse [31,32], with multiple subtypes that present distinct challenges in designing effective prophylactic and therapeutic molecules. While some toxin subtypes differ by as much as 32%, only the C and D serotypes show specific mosaic patterns, which make protection after vaccination particularly challenging. An additional problem with these serotypes has been the marketing of serotype C/D as serotype C, and of serotype D/C as serotype D. Problems relating to vaccine protection against BoNT D led us to pursue additional studies with this toxin, including gene sequencing. As a result, we discovered the subtype discrepancy with BoNT D/C VPI 5995 (from Metabiologics), and verified the subtype of C/D 003-9 (from Wako). Previously, these subtypes were determined to be serotypes D and C solely on immunological information, and since there is some cross-reactivity, even between serotype C1 and serotype D, this has led to confusion as to their true nature. An example of this confusion may be seen in the conflicting reports of cellular toxicity with BoNT C1, which could be due to use of C1 in some experiments and C/D in others [33,34].

To date, all sequenced BoNT C or D strains have shown remarkable conservation to either the standard or mosaic toxins. However, the numbers of published sequences are limited to about 13 BoNT C or C/D strains, and 6 BoNT D and D/C strains. These strains are from Japan, South Africa, Taiwan, and Europe, with only a very few sequences are represented from any specific geographic region. It is possible that as more BoNT C and D strains are sequenced, additional sequence diversity may be seen in these serotypes.

Catastrophic outbreaks of botulism in domestic animals, birds [35,36] and farmed fur animals [12] have had severe environmental and economic impacts. A survey of the literature reveals numerous case studies of botulism outbreaks in cattle, sheep, and goats, where herd losses from 30–77% have been recorded. Losses have been especially hard in South America, Africa, and Australia, where vaccination against BoNT C and D is encouraged. Veterinary use of rBoNT/C1 and /D vaccines could prevent such outbreaks in the future.

Woodward et al. [37] reported that recombinant polyhistidine-tagged rBoNT/C1 and /D Hc antigens produced in *E. coli* were used to inoculate mice as both monovalent and bivalent vaccines. Vaccinations of 10 μg rBoNT/C1 and /D Hc delivered i.p. at 0 and 2 weeks provided 40% and 60% protection against 100,000 LD_{50} of BoNT C1 and BoNT D. The bivalent formula conferred 100% and 40% protection against 1000 LD_{50} challenge with BoNT C1 and BoNT D, respectively. While there was little or no cross protection observed in the monovalent vaccines, mice given the bivalent vaccine survived the BoNT C1 challenge and a subsequent challenge of 1000 LD_{50} of BoNT D. In a separate study, approximately 10 μg of rBoNT/C1 and /D HC expressed as glutathione-*S*-transferase fusion proteins in *E. coli* were used to inoculate mice at 0 and 3 weeks [38] The HC vaccines completely protected mice from challenge with 100,000 LD_{50} of the homologous toxins. No bivalent formulations were investigated in this study. Additionally, the Woodward study did not list the challenge toxin strains used, and the Arimitsu study did not test their vaccines against mosaic strains.

Our rBoNT/C1 and /D Hc vaccines were composed of synthetic genes designed for optimal full-length expression in *P. pastoris*. Surprisingly, both recombinant proteins were cleaved at the same conserved E/Y site (see Fig. 1A and B) by an unidentified protease. The predominant proteolytic product was shown to be stable, despite the inclusion of two significant N-terminal destabilizing amino acids [23]. The relative stability of these recombinant proteins is unexplained but might be attributed to the N-terminal region being sequestered within the recombinant protein, making it unavailable for subsequent degradation.

Results indicate our monovalent rBoNT/C1 and /D Hc vaccines are effective against homologous toxins, and the bivalent formulation can significantly protect against BoNT C1, D and both mosaic toxins. Complete protection was seen after vaccination with vaccines containing similar Hc

sequences as the challenge toxins. Mice vaccinated with rBoNT/C1 Hc were completely protected against BoNT C1 and partially protected against BoNT D/C toxins; mice vaccinated with rBoNT/D Hc were completely protected against BoNT C/D and D toxins. Mice vaccinated with the combination rBoNT/C+D Hc vaccine were completely protected against all C and D toxin subtypes except the BoNT D/C toxin.

Serum ELISA titers reflected survival results. Vaccination with bivalent vaccine resulted in a less robust ELISA titer yet offered the same level of protection against a toxin challenge as the monovalent formulations. While the ELISA titers and survival in the mosaic BoNT D/C-challenged animals appears due primarily to protection from the rBoNT C1 Hc vaccine, the relatively poor ELISA titers and survival rates observed are most likely due to the significant sequence divergence between BoNT D/C, C1 and D observed in the Hc region (Fig. 2). The Hc of this toxin differs by 23% from the BoNT C1 sequence and by 63% from the BoNT D sequence. The antibodies derived from the rBoNT/C1 and /D Hc antigens that do not contain this unique sequence would most likely have a reduced efficacy in neutralizing the D/C mosaic toxin.

One dose potency assays were done with BoNT/C1 Hc vaccination and challenge with BoNT C1 toxin, and with BoNT/D Hc vaccination followed by challenge with either BoNT D or D/C toxin. While the ED_{50} for BoNT/C1 Hc-BoNT C toxin was 114 ng, and the ED_{50} for BoNT/D Hc-BoNT D toxin was 232 ng, there were no survivors when BoNT/D Hc vaccinated mice were challenged with BoNT D/C toxin. The ED_{50} results after challenge with homologous toxin are within the range of ED_{50}s of other rBoNT Hc vaccines (89–250 ng) [1], indicating that equivalent effective protection is seen with all rBoNT Hc vaccines. The potency assays also showed that no effective protection can be gained after single vaccination with rBoNT/D Hc and challenge with mosaic D/C toxin. Woodward et al. indicated that protection might also be limited after two vaccinations. Three vaccinations with our rBoNT /C1 and/D Hc vaccines are time-consuming but effective. These results show our rBoNT/C1 Hc and /D Hc vaccines to be effective against challenge with homologous toxin after single vaccination, but effective protection against heterologous may require a minimum of three vaccinations.

Recombinant BoNT Hc vaccines are known to be highly protective against homologous toxins. This report illustrates their effectiveness in monovalent and bivalent formulations against diverse toxin subtypes.

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